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# Disorder of Sexual Development in a Mare with an Unusual Tentative Mosaic Karyotype: 63,X/64,Xdel(Y)

Stefanie Neuhauser[a] Johannes Handler[a] Claude Schelling[b]
Aldona Pieńkowska-Schelling[c]

[a]Pferdezentrum Bad Saarow, Equine Reproduction Unit, Freie Universität Berlin, Berlin, Germany; [b]Clinic of Reproductive Medicine and Center for Clinical Studies, Vetsuisse-Faculty, University of Zurich, Lindau, and [c]Institute of Genetics, Vetsuisse-Faculty, University of Bern, Bern, Switzerland



**Keywords**
DSD · Horse · Mosaic · Sex chromosome · Y chromosome deletion

**Abstract**
The present report describes a 4-year-old Trakehner mare which was referred to the clinic for a breeding soundness evaluation. Clinical, histological, and postmortem examination revealed an underdeveloped genital tract, the absence of a cervix uteri, and small inactive ovaries without male gonadal tissue. Blood lymphocyte analysis revealed an unusual mosaic karyotype consisting of 2 cell lines. For the majority of cells (70%), monosomy X (63,X) was observed. The remaining cells (30%) contained 64 chromosomes including one X chromosome and a small rudimentary Y chromosome consisting mostly of heterochromatin. The centromere was retained, but its full functionality was questionable. PCR analysis revealed that the entire male-specific region of Y (Yq14), including the *SRY* gene, was deleted. It remained unclear if the pseudoautosomal region (Yq15) and parts of the heterochromatic region (Yq13) were affected by this deletion. The phenotype of the mare with this disorder of sex development associated with sex chromosome abnormalities is genetically comparable to 63,X monosomy which fully explains the clinical findings.


In humans, disorders of sex development (DSD) are defined as congenital conditions in which the development of chromosomal, gonadal, or anatomical sex is atypical [Lee et al., 2006]. Chromosomal aberrations, gene mutations, and environmental factors may disrupt the process of normal sexual development. In domestic animals, a good reproductive performance is an important economic factor and is essential for any breeding program, because a higher reproduction rate results in higher selection intensity and shorter generation intervals, permitting faster genetic progress [Eldridge, 1985]. Recently, Raudsepp and Chowdhary [2016] reviewed the link between chromosomal aberrations and fertility in domestic animals. DSD are a well-known problem in horses, but their prevalence in the general horse popula-

KARGER


E-Mail karger@karger.com
www.karger.com/sxd

Claude Schelling
Clinic of Reproductive Medicine and Center for Clinical Studies
Vetsuisse-Faculty, University of Zurich
Eschikon 27, CH–8315 Lindau (Switzerland)
E-Mail cschelling@vetclinics.uzh.ch

tion remains largely unknown. One larger study encompassing a random sample of 500 young male and female horses in Poland found no aberrations in males and a prevalence of 3.7% for mares with chromosomal aberrations [Bugno et al., 2007]. Based on chromosomal analyses from 204 mares selected for breeding, Nie and coworkers [1993] found a lower prevalence (1.5%) for sex chromosome abnormalities. Power [1990] gives a comprehensive overview of reported chromosomal aberrations in horses up to the year 1990. Since then, numerous cases of intersex horses have been reported, reflecting the advances in the fields of equine genomics and cytogenetics [Raudsepp and Chowdhary, 2016]. Pure monosomy X [Mäkinen et al., 2001; Di Meo et al., 2009; Moreno-Millán et al., 2012] and its mosaic forms [Moreno-Millán et al., 2012; Pieńkowska-Schelling et al., 2016] as well as DSD with sex reversal [Bannasch et al., 2007; Bodvarsdottir et al., 2009; Raudsepp et al., 2010; Villagómez et al., 2011; Peer et al., 2012; Anaya et al., 2014; Pieńkowska-Schelling et al., 2014] are the most common findings in mares with fertility problems [Lear and McGee, 2012]. In addition, translocations [Lear et al., 2008], trisomies [Moreno-Millán et al., 1989; Lear et al., 1999; Mäkinen et al., 1999; Brito et al., 2008], deletions [Halnan, 1985; Raudsepp et al., 2010], isochromosomes [Herzog et al., 1989; Mäkelä et al., 1994; Das et al., 2012], and copy number variations [Ghosh et al., 2014] have been reported less frequently.

The frequency of DSD in the general horse population is most likely underestimated, because a substantial number of affected individuals appear phenotypically normal and are never presented to a veterinarian for clinical examination. Whereas the majority of mares undergoing chromosome analysis have a history of sub-fertility [Pieńkowska-Schelling et al., 2016], ambiguous external genitalia [Bugno-Poniewierska et al., 2014], or deviant behavior [Bodvarsdottir et al., 2009; Peer et al., 2012], some DSD mares are identified on the occasion of breeding soundness examinations [Pieńkowska-Schelling et al., 2014; present case].

The present report describes a phenotypically female horse with gonadal dysgenesis and an unusual mosaic karyotype.

## Materials and Methods

*Animal*

A 4-year-old Trakehner mare (born 2011) showed no signs of heat when teased to different stallions. Upon trans-rectal examination, the private veterinarian diagnosed small ovaries and referred the horse to the clinic. The medical history of the horse showed that she had to be treated for hypogammaglobulinemia and septicemia at a younger age. In addition, an optimized food management was necessary to stop intermittent colic. The horse had to be euthanized within short time after the clinical examination because of a massive and rapid deterioration of health, and a postmortem examination of the internal genital tract became possible. However, the owner did not agree to a dissection of other organs or any additional genetic testing.

*Cytogenetic and Molecular Analyses*

Heparin- and EDTA-treated blood samples were collected for cytogenetic and molecular analysis, respectively. Chromosomes were prepared according to standard protocols from short-term lymphocyte cultures. Giemsa staining was applied to count the chromosomes. DAPI- and CBG-banding of metaphase chromosomes followed the methods described by Schweizer [1980] and Sumner [1972], respectively. More than 200 metaphases were analyzed and karyograms were prepared. FISH with an in-house developed equine whole Y chromosome painting probe [according to Pieńkowska-Schelling et al., 2006] was applied to identify the sex chromosomes. This painting probe hybridizes along the entire acrocentric Y chromosome and to the heterochromatic block of the X chromosome (Xq17q21). However, it does not detect the pseudoautosomal region (PAR) on Xp. Telomeres were analyzed with FISH using a commercially available human telomeric probe (TEL100R, ChromBios, Germany) according to the manufacturer's protocol. The results of cytogenetic and FISH experiments were analyzed with a Zeiss Axio Imager Z1 microscope and Ikaros/Isis-software (MetaSytems GmbH, Germany). High molecular weight genomic DNA was extracted by a proteinase K/phenol extraction method. PCR amplification of the *SRY* gene from genomic DNA was performed using a primer pair described in Han et al. [2010], which results in a 714-bp amplification product. Additional PCR primers for the equine Y chromosome (ECAY) contigs [Paria et al., 2011] were used: *YM2* (contig I, 119 bp) [Wallner et al., 2004], *YE1* (contig II, 199 bp) [Wallner et al., 2004], *NLGN4Y* (contig III, 156 bp) [Paria et al., 2011], *AMELY* (contig IV, 160 bp) [Hasegawa et al., 2000], *ZFY* (contig V, 342 bp) [Lindgren et al., 2001].

## Results

*Clinical Examination*

On the day of the presentation of the mare, there were no specific clinical findings with the exception of atrophic muscles in the croup. Examination of the external genitalia revealed a vulva with a vertical position and a Caslick index <50. The total vulvar opening was 5 cm in length and the ventral part of the vulva did not seal appropriately. Trans-rectal palpation and ultrasonography revealed very small ovaries without follicular development. The estimated length, width, and height of the left and right ovary were 10 × 5 × 5 and 10 × 10 × 5 mm, respectively. The uterus was also very small (estimated diameter of uterus horns was 10 mm) with a homoge-

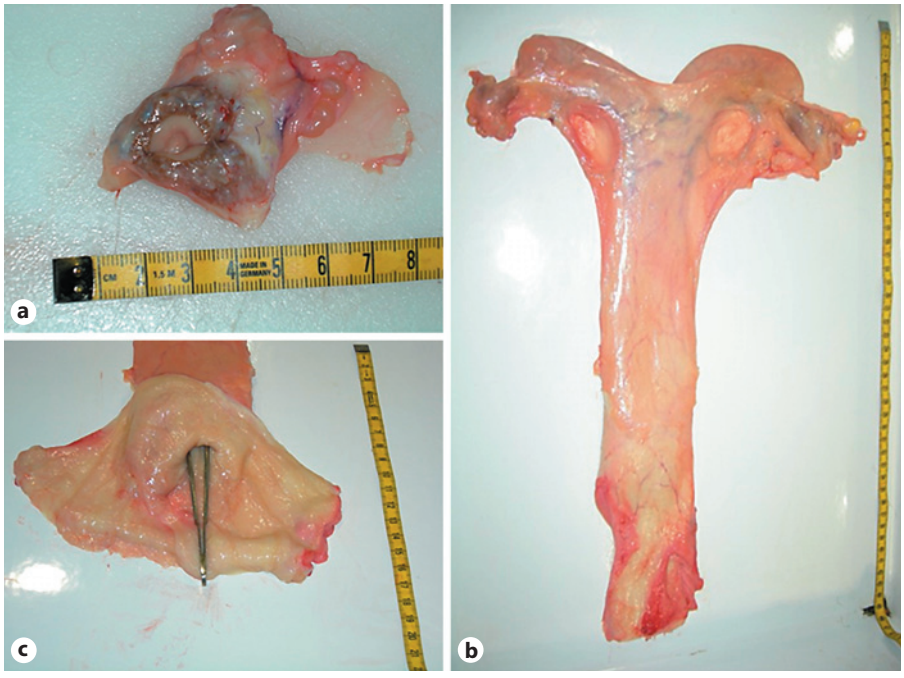


**Fig. 1. a** Longitudinal section of the left ovary showing absence of follicles. **b** Dorsal view of the uterine body with short uterine horns. **c** Absence of a uterine cervix.

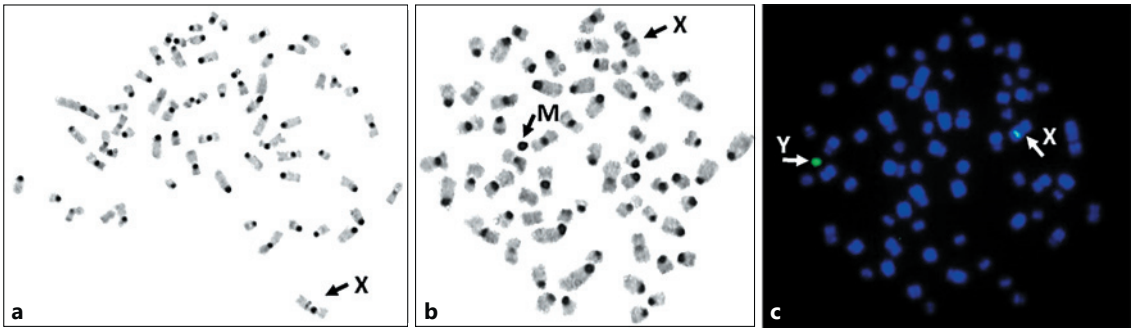


**Fig. 2.** Three metaphases of the mare with DSD. CBG-banded metaphases with karyotype 63,X (**a**) and 64,X+mar (**b**). Arrows indicate the X chromosomes and the marker chromosome (M). **c** Metaphase with karyotype 64,X+mar after hybridization with a whole equine Y chromosome painting probe. Arrows indicate the marker chromosome and the heterochromatic band on the long arm of the X chromosome (Xq17q21).

neous echotexture. It was not possible to verify the existence of the cervix uteri. The vagina appeared to be normal. These results were confirmed by a postmortem examination of the genital tract. Both ovaries were very small without growing follicles (Fig. 1a), and histologically no testicular tissue could be seen (not shown). The uterus was underdeveloped with very short uterine horns (Fig. 1b), and the cervix was not developed (Fig. 1c). Histology of the endometrium revealed the presence of few uterine glands and no signs of fibrosis or inflammation (not shown).

*Cytogenetic and Molecular Analysis*

After Giemsa staining, CBG- and DAPI-banding of metaphase chromosomes from blood lymphocytes, 2 distinct cell lines were obvious. CBG-banding showed that in 70% of the cells only 63 chromosomes including 1 X chromosome were visible (63,X) (Fig. 2a). In the remaining cells, we observed 63 chromosomes (including an X chromosome) and an additional marker chromosome (64,X+mar) (Fig. 2b). The autosomes appeared normal with no visible gross structural aberrations. CBG-banding showed that the marker chromosome consisted of

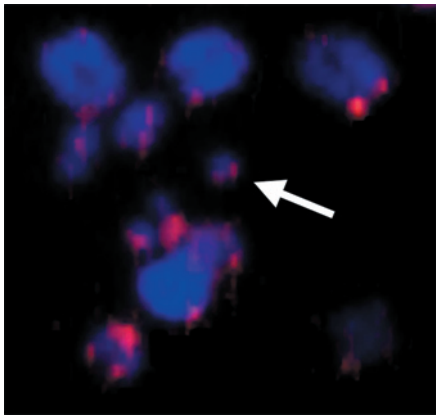

**Fig. 3.** Partial metaphase with telomeric signals (counterstained with DAPI) of the cell line with the marker chromosome. Arrow points to the distal end of the rudimentary Y chromosome.

heterochromatin only (Fig. 2b), similar to the equine Y chromosome. Therefore, an equine whole Y chromosome painting probe was hybridized to metaphase chromosomes. In the 64,X+mar cell line clear signals were seen on the entire length of the marker, proofing its Y origin and along the heterochromatic band of Xq17q21 (Fig. 2c). In the 63,X cell line, signals were restricted to the heterochromatic band of the X chromosome (not shown). Because of the known extensive length variation of the equine Y chromosome [Power, 1988], it is difficult to make a statement about the size of the rather small marker chromosome. It cannot be ruled out that parts of the heterochromatin were affected by the deletion. Therefore, it is not possible to determine the exact breakpoints of the deletion. FISH signals with a human telomeric probe (25 spreads analyzed) were observed only at the distal end of the marker chromosome (Fig. 3). Based on the irregular segregation of this marker chromosome, without other scientific proof, we suspect that its centromere is not fully functional. Taking the cytogenetic results together, the karyotype of the mare is best described by 63,X/64,Xdel(Y).

PCR analysis revealed no amplification of a PCR product with the expected length of 714 bp for *SRY*. However, there were 2 shorter amplification products visible (not shown) with a length of ca. 580 and 260 bp, which were sequenced, analyzed by BLAST (EquCab2.0 sequence), and matched with NC_009167.2 (ECA24) and NC_009154.2 (ECA11), respectively. There was no amplification of *YM2*, *YE1*, *NLGN4Y*, *AMELY*, and *ZFY*, representing contigs I, II, III, IV and V of the equine male-specific region of the Y (MSY), respectively (not shown).

## Discussion

Disorders of sex development are a major cause of infertility and congenital abnormalities in the horse [Lear and McGee, 2012], and consequently, reproductive failure and sexual ambiguity are among the foremost reasons for performing chromosome analyses in this species. Pure monosomy X and its mosaic forms as well as male-to-female sex reversal are the most common findings in mares with gonadal dysgenesis [Lear and McGee, 2012], but the former are rather rarely reported in other domestic species. In horses, including the present case, numerical and structural chromosomal abnormalities involving the equine X chromosome are often associated with mosaic karyotypes [Durkin et al., 2011]. The acrocentric equine Y chromosome is quite particular. It is mainly composed of heterochromatin and the euchromatic region is located distally on the long arm with a rather small PAR region. It has been suggested that the size of the PAR itself might be related to the differences in frequencies for X mosaicism observed between species [Raudsepp et al., 2012]. In addition, in contrast to the situation in humans, the equine *SRY* gene is located proximally of the MSY, far from the PAR region [Raudsepp et al., 2004].

Based on the results, the mare presented in this report showed a form of DSD associated with abnormalities of the sex chromosomes. The entire internal genital tract of the mare was underdeveloped with small inactive ovaries, and a cervix uteri was not detectable. Histologically, there was no evidence for the presence of testicular tissue in the ovaries. Cytogenetic analyses of blood lymphocytes revealed an unusual mosaic karyotype consisting of 63,X and 64,Xdel(Y) cells with frequencies of 70 and 30%, respectively. It has to be stressed that these findings may not be representative for other tissues like it has been shown for intersex horses before [Höhn et al., 1980].

The majority of cells analyzed for the mare showed monosomy of the X chromosome which results in haploinsufficiency of PAR and non-PAR genes that escape X inactivation. The second cell line of the mare carried a rudimentary Y chromosome in which the entire MSY, and most likely also the PAR region, were deleted. Again, there is a dosage problem for the PAR genes. PCR analysis was applied to characterize the size of the deletion. It confirmed the deletion of the entire male-specific part of the Y chromosome, including the *SRY* gene. Furthermore, without experimental prove, we assumed that the genes of the PAR were deleted, too. Using primer pairs for the *SRY* gene, 2 clear amplification products of shorter length were detected. Because we never observed these

PCR products from DNA of stallions or geldings, they were further characterized. The PCR primers for the *SRY* gene seem to bind to 2 similar binding sites present on equine chromosomes 11 and 24, respectively. This amplification probably may have resulted just from the lack of true binding sites on the Y chromosome which are not present in mares or in intersexes like the present case. To clarify a possible biological consequence, additional work is needed.

Therefore, based on the cytogenetic and molecular results, the present horse should exhibit the phenotype of a mare with a pure 63,X chromosomal constitution.

In humans, 45% of Turner patients show full aneuploidy 45,X, and the remaining 55% consist of 45,X mosaics with or without a Y chromosome [Zhong and Layman, 2012; Ackermann and Bamba, 2014]. However, the existence of pure 45,X individuals, for at least in humans, has been challenged. Because sex chromosome monosomies 45,X or 45,Y are considered not to be compatible with life, it has been suspected that individuals with full 45,X aneuploidy might be cryptic mosaics for which the detection of the 46,XX cell line is difficult [Hook and Warburton, 2014]. Interestingly, in our certainly restricted clinical material consisting of 46 mares, which were karyotyped for subfertility (19), breeding soundness examination (15), deviant behavior (5), malformations (4), and abnormalities of the genital tract (3), we never observed a full monosomy X [Schelling, unpublished].

Despite of the important function of the Y genes for male fertility, relatively few chromosomal aberrations have been reported for domestic species [Raudsepp and Chodhary, 2016]. In humans, Y microdeletions are frequently found in men with fertility problems [Colaco and Modi, 2018]. Large deletions of the Y chromosomes are rarely reported and may result in infertile men with some manifestations of Turner syndrome [Fitch et al., 1985].

Several cases of 65,XXY or 65,XYY cell lines in conjunction with a 63,X or a 64,XX cell line have been reported in horses [Bouters et al., 1975; Höhn et al., 1980; Paget et al., 2001]. In addition, similar but distinct mosaic karyotypes including a 63,X cell line have been reported in intersex horses. Bugno et al. [2008] presented a case of a male pony with 3 blood cell lines 63,X, 64,XX, and 65,XXdel(Y)(q?). The deletion of the Y chromosome was comparable with the one seen in the present case, resulting in a loss of genes of the MSY and probably in haploinsufficiency of the PAR genes. However, this horse had a small penis inside the vulva and small inguinal testes, indicating an effect through a *SRY* gene product during development. Two other chromosomally mosaic horses with a Y isochromosome (Yq) in conjunction with a 63,X cell line were reported [Herzog et al., 1989; Das et al., 2012]. Both cases had the Y isochromosomes which were composed of 2 long arms, resulting in genetic Y disomy and overdose of PAR genes. Interestingly, the cells carrying the Y isochromosome strongly differed in their frequencies (89 vs. 4%) without big phenotypic differences. Another report on 18 mares demonstrated molecular heterogeneity of sex reversal in horses [Raudsepp et al., 2010]. Two of these mares (*SRY*-negative) with gonadal dysgenesis had a large deletion of the Y chromosome, removing the entire euchromatic and PAR region in one case and contig I as well as parts of heterochromatin (proximal of contig I) in the other case. Their phenotype regarding the internal genital tract was similar to the mare presented here, and, noteworthy, one mare had a malformation of the cervix uteri. However, these 2 mares were not mosaics and had normal numerical 64,XY karyotypes. The authors suggested that the deletions are the result of interchromatide recombination events between repeated sequences of the Y chromosome, leading to deletions and duplications. In the case of the present mare, we speculate that a normal oocyte of her mother with 31 autosomes and an X chromosome was fertilized by a sperm carrying this rudimentary Y chromosome. The lack of a *SRY* gene product guided the embryo into a female direction. Because of the not fully functional centromere of the rudimentary Y chromosome, its segregation was disturbed and led to a loss in part of the cells and mosaicism.

The clinical findings as well as postmortem and histological examinations of the uterus and gonads together with the cytogenetic and molecular results confirm the conclusive diagnosis of a DSD associated with sex chromosome abnormalities. Although chromosomal aberrations are known to reduce fertility in horses, awareness among breeders and veterinarians for cytogenetic analyses allowing for an optimized diagnosis and reliable prognosis on fertility in problem mares is still poor. Hence, analysis of chromosomes along with molecular analyses of sub-fertile mares should always be taken into consideration for an encompassing breeding soundness examination.


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## Statements of Ethics

The authors have no ethical conflicts to disclose.

## Disclosure Statement

The authors have no conflicts of interest to declare.

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